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The formation of brown adipose tissue induced by transgenic over-expression of PPARγ2

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ABSTRACT

Brown adipose tissue (BAT) is specialized to dissipate energy as heat, therefore reducing fat deposition and counteracting obesity. Brown adipocytes arise from myoblastic progenitors during embryonic development by the action of transcription regulator PRDM16 binding to PPARγ, which promotes BAT-like phenotype in white adipose tissue. To investigate the capability of converting white adipose tissue to BAT or browning by PPARγ *in vivo*, we generated transgenic mice with over-expressed PPARγ2. The transgenic mice showed strong brown fat features in subcutaneous fat in morphology and histology. To provide molecular evidences on browning characteristics of the adipose tissue, we employed quantitative real-time PCR to determine BAT-specific gene expressions. The transgenic mice had remarkably elevated mRNA level of UCP1, Elovl3, PGC1α and Cebpα in subcutaneous fat. Compared with wild-type mice, UCP1 protein levels were increased significantly in transgenic mice. ATP concentration was slightly decreased in the subcutaneous fat of transgenic mice. Western blotting analysis also confirmed that phosphorylated AMPK and ACC proteins were significantly ($P < 0.01$) increased in the transgenic mice. Therefore, this study demonstrated that over-expression of PPARγ2 in skeletal muscle can promote conversion of subcutaneous fat to brown fat formation, which can have beneficial effects on increasing energy metabolisms and combating obesity.

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1. Introduction

Adipose tissue functions as the storage of lipids and energy metabolism in animals. Adipose tissues are mostly made of white adipose tissue (WAT), which primarily serves to store extra energy in the form of triglycerides. Besides WAT, there is the other type of adipose tissue known as brown adipose tissue (BAT), which is specialized to use oxidative phosphorylation to dissipate energy as heat to reduce fat deposition, thereby counteracting obesity [1,2]. BAT is more like skeletal muscle tissue, having abundant mitochondria and functions as the adaptive thermogenesis of the body. The color of the BAT is brown and organized as multiple small lipid droplets, which have a high content of mitochondria. Heat generation due to mitochondrial uncoupling from brown adipose tissue is activated whenever the extra heat is needed. When brown adipose tissue is in active states, stored lipids and glucose are combusted for heat generation in the tissue [3].

The lipid metabolism in BAT, unlike skeletal tissue, primarily utilized uncoupling protein-1 (UCP1) pathway to generate heat energy. UCP1 is expressed almost exclusively in BAT. It is a transporter protein in mitochondrial transmembrane which regulates the protein transport and has a high oxidative capacity, thus controlling fat acids (FAs) oxidation and generating energy expenditure [4]. UCP1 is usually located in the mitochondrial inner membrane of brown adipose tissue, regulating adaptive thermogenesis by uncoupling respiration in BAT and engendering heat instead of ATP. It is believed that UCP1 enhances glucose uptake via AMPK activation. AMPK acts as an intracellular energy sensor to adjust the rates of metabolic pathways of ATP production [5,6]. Phosphorylated AMPK inactivates acetyl-CoA carboxylase (ACC) through phosphorylation, an enzyme that catalyzes arate-limiting step in fatty acid synthesis pathway [7,8]. Level of phosphorylated AMPK, along with ACC phosphorylations, serves as indicator of reduced energy storage. Brown fat recruitment referred to the induction of increasing brown adipose tissue activity. Cold stimulation can induce brown adipocytes in retroperitoneal fat of adult A/J mice [9]. Because of its thermogenesis, brown adipose tissue with its characteristic protein-UCP1, may be beneficial to neonatal

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survival [3]. BAT was assumed to work in young mammals, and had little effect in adults [10]. However, distinct BAT deposits were reported in adults, and especially had stronger activity in non-obese adults [11]. Therefore, BAT can be a therapeutic target to combat obesity [12].

In BAT, a rapid induction of UCP1 and its upstream regulator peroxisome proliferator activated receptor gamma coactivator (PGC1α) can regulate non-shivering thermogenesis at reduced temperatures [13,14]. PGC1α is highly expressed in tissues, which have abundant mitochondria and active oxidative metabolism such as brown adipose tissue and skeletal muscle, whereas the expression of PGC1α is low in liver and white adipose tissue [13]. PGC1α is involved in the brown adipocyte differentiation process by binding to peroxisome proliferator-activated receptor-γ (PPARγ) and coactivating PPARγ to stimulate the transcription of several genes specific in BAT [14].

PPARγ nuclear receptor is an essential regulator of lipid, glucose, and insulin metabolism [15]. The receptor is expressed in mice and humans as two different isoforms, PPARγ1 and PPARγ2 [16]. PPARγ2 differs from PPARγ1 by 30 additional amino acids on its N-terminus. As the master regulator of fat-cell formation, PPARγ2 is required for the accumulation of adipose tissue and hence contributes to obesity [17]. PPARγ2 is necessary and sufficient to induce adipogenesis. However, its expression in muscle is only 5–10% of fat [18,19]. Skeletal muscle-specific PPARγ deletion causes severe insulin resistance, with milder defects observed in adipose tissue and liver [20]. Studies from muscle cell culture also showed that determined myoblasts with no inherent adipogenic potential can be induced to transdifferentiate into mature adipocytes by the ectopic expression of PPARγ and C/EBP alpha [21]. Furthermore, over-expression and activation of PPARγ2 was previously shown to promote adipogenic conversion of the myoblast [22,23]. Recent studies have demonstrated that brown adipocytes arise *in vivo* from precursors that express Myf5 as well. It can convert myogenic cells into adipocytes but PRDM16 expression additionally commits cells to the brown fat fate [24]. To investigate the capability of converting white adipose tissue to BAT or browning by PPARγ *in vivo*, we generated transgenic mice with tissue-specific expression of PPARγ2 under the control of muscle creatine kinase promoter. The transgenic mice showed strong brown fat features in the subcutaneous fat in morphology and histology. The results demonstrated that over-expression of PPARγ2 promote BAT formation in transgenic mice.

2. Materials and methods

2.1. Transgene construct and transgenic animals

The vector pEGFP-N1 Ls MCK-sPPARγ2 was constructed according to Huang et al. [25] and used for pronuclear injection. TG mice were generated by a standard DNA microinjection [26,27]. Founder TG mice were allowed to mate with wild-type mice (C57BL6/J strain) and give birth to the F1 generation of TG mice, which were used and compared with littermate WT mice in all experiments. All mice were kept at room temperature (22 °C) with a 12 h light/dark cycle. Mice were ad libitum fed a chow diet. All procedures were in accordance with institution guidelines and approved by the Institutional Animal Care and Use Committee of Hubei Province.

2.2. HE histology and immunohistochemistry

Subcutaneous fat was fixed in 4% paraformaldehyde, embedded in paraffin and cut into 4 μm thick sections. Sections were stained with hematoxylin and eosin. In addition, the anti-UCP1 antibody (1:100 dilutions, Abcam) in immunohistochemistry was performed according to SABC immunohistochemical staining kit (Boster, China).

Table 1
Primers used for real-time PCR.

Gene	Forward primer	Reverse primer
β-Actin	AGGCCCAGAGCAAGAGAGGTA	GGGGTGTTGAAGGTCTCAAACA
Cidea	TGCTCTTCTGTATCGCCCAGT	GCCGTGTTAAGGAATCTGCTG
Adiponectin	GCACTGGCAAGTTCTACTGCAA	GTAGGTGAAGAGAACGGCCTTGT
Cox8b	GAACCATGAAGCCAACGACT	GCGAAGTTCACAGTGGTTCC
Elovl3	TCCGCGTTCTCATGTAGGTCT	GGACCTGATGCAACCCTATGA
Glut4	CTGTCGCTGGTTTCTCCAACT	CCCATAGCATCCGCAACATA
PPARα	AGAGCCCCATCTGTCCTCTC	ACTGGTAGTCTGCAAAACCAAA
PRDM16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG
UCP1	ACTGCCACACCTCCAGTCATT	CTTTGCCTCACTCAGGATTGG
Dio2	CAGTGTGGTGCACGTCTCCAATC	TGAACCAAAGTTGACCACCAG
Cebpα	TGCGCAAGAGCCGAGATAAA	CCTTCTGTTGCGTCTCCACG
Fabp4	ACAAGCTGGTGGTGGAATGTG	CCTTTGGCTCATGCCCTTT
PGC1α	CCCTGCCATTGTTAAGACC	TGCTGCTGTTCCTGTTTTC
PPARγ2	TCCCGCTGACCAAAGCAAAGGC	CCACGGAGCGAAACTGACACCC

2.3. Gene expression analysis by real-time PCR and Western blotting

Total mRNA was isolated from subcutaneous fat using the RNeasy Lipid Tissue Mini kit (Qiagen, Germany). Complementary DNA was prepared from total RNA using the Revert Aid TM First Strand cDNA Synthesis Kit (MBI Fermentas, Lithuania, USA) after DNase digestion of possible genomic DNA in the samples. Real-time PCR was performed with the SYBR qPCR Mix (Toyobo, Osaka, Japan) in a Bio-Rad CFX96 Real-Time PCR system. The primers sequences refer to the literature Seale [26] and Sun [28], listed in Table 1. Relative gene expression levels were calculated by ΔΔCt method using β-actin as control [29]. Detections of UCP1, p-AMPK, AMPK, ACC and pACC protein were carried out by Western Blotting as described by Huang [25]. Rabbit anti-UCP1 (1:1000) and anti-β-actin (1:1000) were obtained from Abcam (Cambridge, MA). Rabbit anti-p-AMPKα1/2 antibody (Thr 172, 1:100 dilution) and anti-AMPK antibody (1:1000 dilution) from Santa Cruz Biotech, and Rabbit anti-p-ACC (1:400) and anti-ACC (1:400) antibodies were from Cell Signaling Technology were used. Mouse anti-α-Tubulin was obtained from Beyotime (China) in the Western blotting. Vision Works LS software from UVP was used for quantifications of band density.

2.4. Fluorescent detection of ATP

Six two-month-old mice were used for analyzing mitochondrial ATP. Mitochondrial ATP was prepared through ATP Assay Kit (Beyotime, China) according to the manufacturer's instructions. ATP fluorescent levels were measured in Promega GloMax-Multiple Base Instrument with Luminescence Module.

2.5. Statistical analysis

Data are expressed as means ± SEM. We use unpaired 2-tailed Student's *t*-test to evaluate statistical significances. $P < 0.05$ was considered as statistical significant.

3. Results

3.1. Brown fat formation in the PPARγ2 transgenic mice

Five founder transgenic mice were obtained by a standard DNA microinjection (Line 12, 38, 41, 59 and 61). There was no difference in body weights between MCK-PPARγ2 transgenic mice and wild-type mice (Fig. 1). Observations of subcutaneous fat indicate

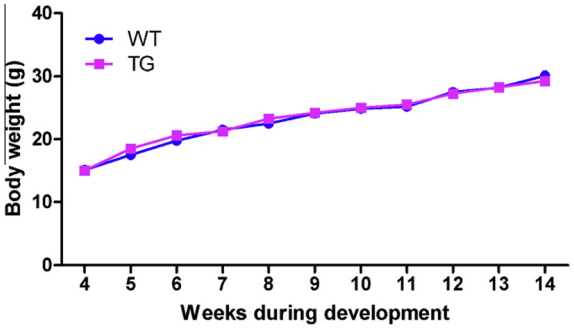

Fig. 1. Growth curve of wild-type and MCK-PPARγ2 transgenic mice is plotted by weekly body weight for eight weeks. Each time point is based upon the analysis of 5–8 mice.

BAT-like phenotype or browning in TG mice compared with WT mice at two months of age (Fig. 2A). Histological analysis illustrated distinct differences in the morphology and appearances of the subcutaneous fat derived from TG with unilocular adipocytes in fat pad. Small fat particles and more nucleo-cytoplasmic appeared in the subcutaneous fat of transgenic mice. By using rabbit anti-UCP1 antibody as the primary antibody, immunohistochemistry result shows TG mice have increased UCP1 protein levels in the transgenic mice compared with wild-type mice (Fig. 2B). These results supported the browning features or formation of BAT in the transgenic mice.

3.2. Expression analysis of selective brown fat-specific genes and adipocyte genes

Real-time PCR for gene expression analysis was performed with adipose tissue from two months old mice. The results demonstrated that UCP1 and PPARγ2 mRNA levels were significantly ($P < 0.05$) increased in TG mice in comparison with wild-type mice by 14.1 fold and 56.4 fold, respectively (Fig. 3A). Protein levels analyzed by Western blot also showed that UCP1 was increased remarkably in TG mice (Fig. 3B), which is consistent with the qPCR results. Real-time PCR analyses of several BAT and adipocyte marker genes in subcutaneous fat were examined in 2-months old mice. A significant increase was observed in the relative BAT marker gene expression of PRDM16, Elovl3, PGC-1α, Cidea, PPARα and Dio2 (Fig. 3C). The fold changes ranged from 2 to 14. TG mice also showed significant up-regulation of adipogenic markers common in brown and white fat, including glucose transporters 4 (Glut4) by 1.8 fold, CCAAT/enhancer binding protein alpha (Cebpα) by 1.5 fold and fatty acid binding protein 4 (Fabp4) by 2.15 fold (Fig. 3D). Adiponectin and Cox8b gene expression by qPCR analysis

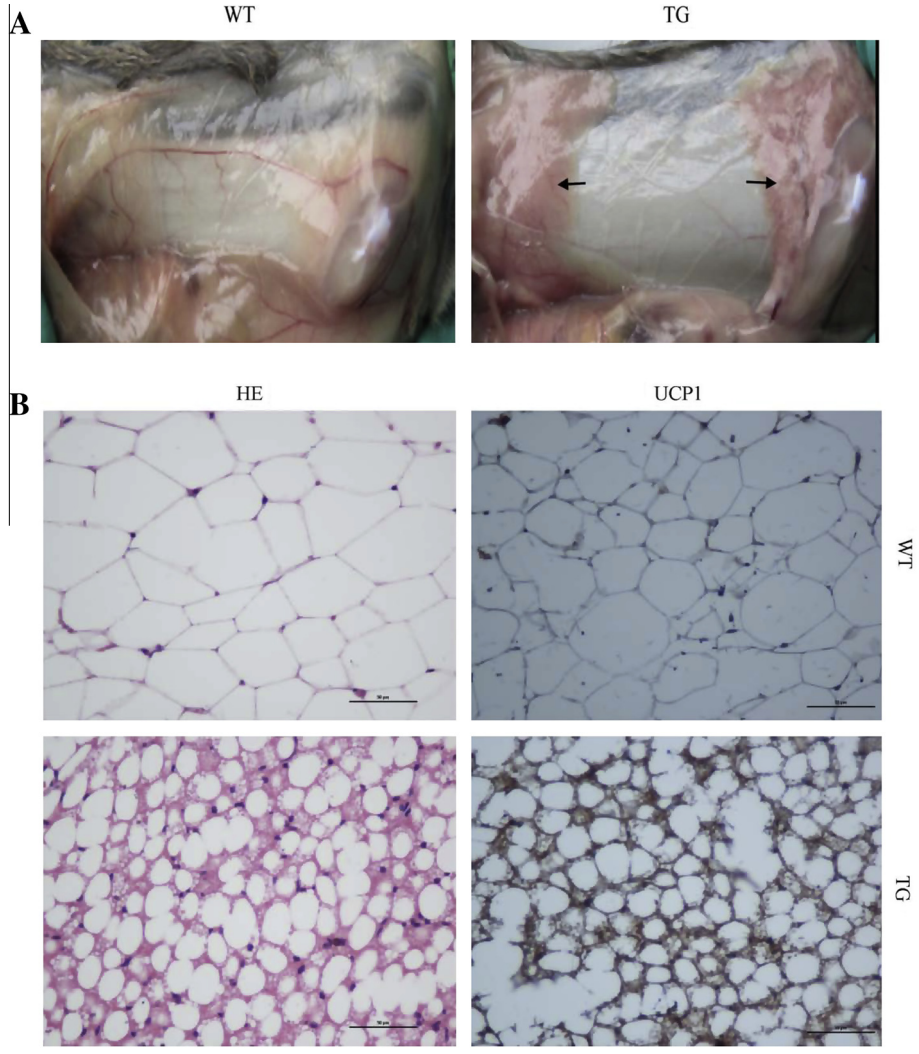

Fig. 2. Formation of BAT in subcutaneous fat. (A) Induction of BAT formation in subcutaneous fat by transgenic expression PPARγ2 at two months of age. Arrows point to the brown adipose tissue in transgenic mice. (B) H&E and Immunohistochemistry for UCP1 of subcutaneous fat of WT and TG mice. Magnification: ×40.

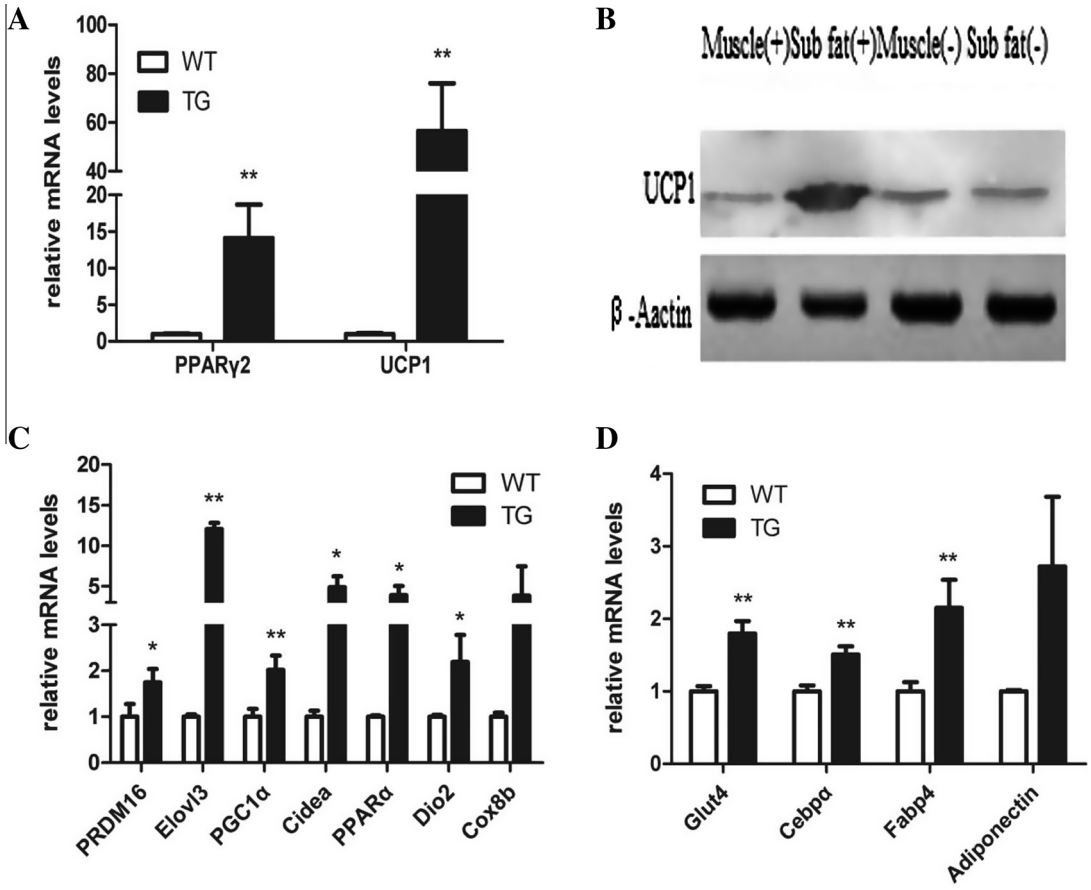

Fig. 3. Results of gene expression analysis. (A) Real-time PCR analysis of UCP1 and PPARγ2 gene expression levels in subcutaneous fat. (B) Analysis of UCP1 protein expression from soleus muscle and subcutaneous fat by Western blot with β-actin as reference for equal loading. (C) Gene expression levels of BAT-specific genes in subcutaneous fat from two-month-old mice. (D) Gene expression levels of adipocyte genes in subcutaneous fat from two-month-old mice. (Values are presented as means ± SEM; $n = 3$; $^{*}p < 0.05$ $^{**}p < 0.01$).

increased, but did not reach significant level in transgenic mice compared with wild-type mice (Fig. 3C and D).

3.3. Activation of AMPK in subcutaneous fat through reduction of ATP production

Mitchondrial ATP concentration in subcutaneous fat was slightly decreased in transgenic mice (1.54 μmol/L) compared with wild-type mice (1.67 μmol/L). There was no difference in mitochondrial ATP concentration in interscapular brown fat between transgenic mice and wild-type mice (Fig. 4A). Then, we tested whether the decreased ATP concentration was a consequence of UCP1-mediated AMPK activation. By using p-AMPKα ½ antibody (Thr172) and p-ACC antibody in Western blotting, we found that PPARγ2 transgenic mice had much higher levels of phosphorylated AMPK and ACC protein compared to the wild-type littermate control group ($P < 0.01$, Fig. 4B and C).

4. Discussion

PPARγ2 expression is restricted to adipocytes and essential for differentiation and maintenance of adipose tissue function. Activation of PPARγ with thiazolidinediones (TZDs) induces a brown phenotype in white adipocytes *in vivo* and *in vitro* by induction of brown-specific genes and repression of a set of white fat genes [30]. In addition, SirT1-dependent PPARγ2 deacetylation promotes browning of subcutaneous WAT by regulating ligand-dependent coactivator/corepressor exchange at the PPARγ2 transcriptional complex [31]. The transcriptional regulator PRDM16 controls a bidirectional cell fate switch between skeletal myoblasts and brown fat cells. The only DNA-binding transcriptional component found in the PRDM16 complex was PPARγ2. It can convert myogenic cells into adipocytes but PRDM16 expression additionally commits cells to the brown fat fate. Although PPARγ alone can drives adipogenesis, does not induce the brown fat cell gene program in C2C12 myoblasts [24].

In consideration of the specific relationships in BAT, WAT and PPARγ, we hypothesized that the over-expressed PPARγ can induce "browning" in WAT. In the current study, we investigated the browning characteristics of WAT with the transgenic mice. The results showed that there was no difference of body weight between TG and WT mice. In the mice with over-expressed UCP1 in skeletal muscle there is no significant difference in food intake, but body weight was significantly lower than the control group and increased insulin sensitivity [32]. We believe that increased thermogenes is may not have significant effects on body mass since energy homeostasis is tightly regulated [33]. In addition, transgenic over-expression of PPARγ2 results in brown fat feature in subcutaneous fat tissue. It showed notable phenotypic and histological changes between TG and WT mice. The results demonstrated that the PPARγ2 transgenic mice had stimulated subcutaneous fat part into brown fat. The increased formation of BAT is mostly observed in subcutaneous fat. We believe the browning feature in subcutaneous fat may be initiated during muscle and adipose tissue formation during fetal development in utero. The

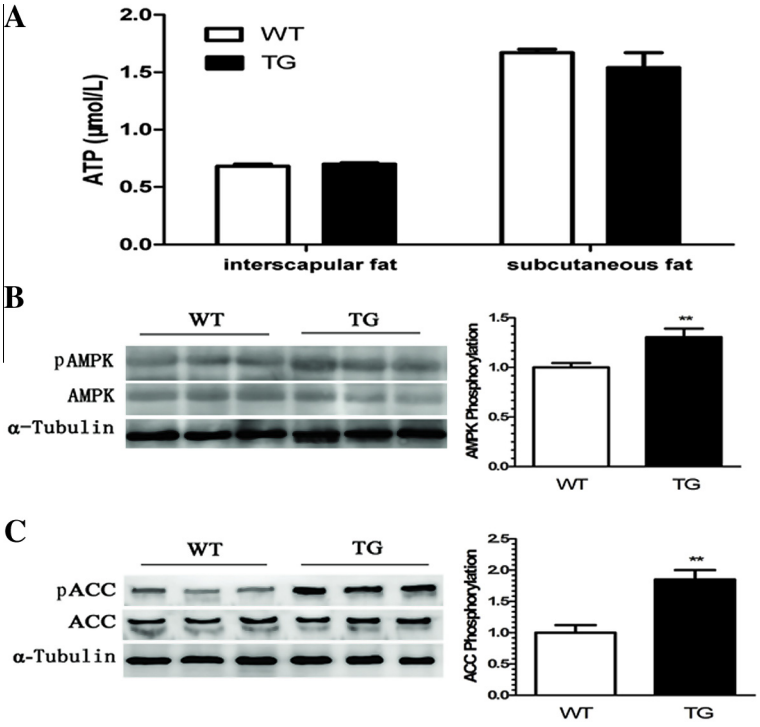

Fig. 4. Mitochondial ATP level and phosporylated AMP-activated protein kinase (pAMPK) protein immnoblots in subcutaneous fat. (A) Relative mitochondrial ATP levels in subcutaneous fat ($n = 3$ for each genotype). (B) AMPK T-172 phosphorylation and AMPK total protein in subcutaneous fat of the TG and WT mice were assayed by Western blotting. AMPK phosphorylation was determined as phospho-AMPK normalized by total AMPK protein. (C) ACC phosphorylation and ACC total protein in subcutaneous fat of the WT and TG mice were assayed by Western blotting. (Values are presented as means ± SEM; $n = 3$; $^{**}p < 0.01$).

browning feature is also likely an indirect effects of the PPARγ2 transgene expression as a result of increased adipose tissue formation in muscle tissue as the mice did have interested intramuscular fat.

Consistent with the function of UCP1 gene, the results demonstrated an increased level of UCP1 mRNA and protein levels. UCP1 regulates energy metabolism and influences the body energy balance, basal metabolic rate and food conversion efficiency. BAT dispersed in a small amount of human adipose tissue and UCP1 mRNA in intra-abdominal fat of obese was about 50% of non-obese [34]. The gene expression results are consistent with norepinephrine and the peroxisome proliferator-activated receptors (PPAR)-ligand rosiglitazone can synergistically regulating Elovl3 gene expression in brown adipocytes [35,36]. The commonly used BAT-enriched markers include PRDM16, Elovl3, PGC-1α, cell death-inducing DNA fragmentation factor alpha subunit-like effector A (Cidea), peroxisome proliferator activated receptor alpha (PPARα), deiodinase iodothyronine type II (Dio2) and Cox8b. Cidea expression is largely restricted to BAT and controlled by PPARγ [37]. It is a mitochondrial protein that negatively regulates the activity of UCP1 in BAT uncoupling protein UCP1 [38]. PPARα are mainly present in BAT, liver, kidney, and heart and characterized by relatively high rates of fatty acid catabolism in adult mice [39]. Dio2 in BAT are related to cold-stimulated T3 production through PGC-1α signaling pathways [13]. The disruption of Dio2 results in BAT-specific hypothyroidism [40]. Cox8b is a subunit of cytochromec oxidase that is induced during mitochondrial biogenesis [41]. We showed that selective BAT marker genes expression were significantly increased, including UCP1, Elovl3, PGC1α and Cebpα mRNA levels.

AMPK plays a critical role in maintaining energy balance, which functions as an energy sensor in many tissues, including skeletal muscle [42]. AMPK can be activated by an increase in intracellular AMP/ATP ratio during a low-energy state and by phosphorylation at Thr172 in the catalytic α-subunit [43]. AMPK is stimulated by cellular stresses that deplete ATP and elevate AMP such as diet restriction, hypoglycemia, exercise [44]. AMPK modulates mitochondrial gene expression seem to require PGC-1a, either by increasing its expression or direct phosphorylation [45]. Compared with wild-type mice, ATP concentration slightly decreased in the subcutaneous fat of the transgenic mice, which is supported by the increased levels of AMPK phosphorylation, along with increased ACC phosphorylation in the transgenic mice. The mitochondria in BAT operate in an uncoupled mode via UCP1-they activate AMPK pathway to reduce markedly ATP production to maintain energy balance, and increase fatty acids oxidation of fatty acids to produce heat. The phosphorylated AMPK and ACC data provides strong evidences of reduced energy storage as a result of increased energy expenditure through increased UCP1 expression and conversion of WAT to brown adipocyte in the transgenic mice.

Based on the brown fat features in the PPARγ2 transgenic mice, we concluded that PPARγ2 mediates a significant part of energy metabolism between skeletal muscle and adipose tissue. Promoting BAT features in white adipocytes has therapeutic potential to prevent obesity and diabetes. The existence of brown fat cells in white adipose tissue and the reported transdifferentiation from white into "browning" adipocytes may open a new way to increase metabolic rate as a way to combat or prevent obesity.

Acknowledgments

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